Glycoprotein 60 Diversity in Cryptosporidium parvum Causing Human and Cattle Cryptosporidiosis in the Rural Region of Northern Tunisia

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Abstract. The zoonotic potential of Cryptosporidium parvum was studied in an extensive cattle farming region of northern Tunisia. Seventy fecal samples from pre-weaning calves and 403 fecal samples from children were examined by microscopy after modified Ziehl–Neelsen (MZN) staining. Positive Cryptosporidium specimens were identified at a species level using an 18S rRNA nested polymerase chain reaction (PCR) followed by an Restriction Fragment Length Polymorphism (RFLP) analysis. C. parvum isolates were subgenotyped by sequence analysis of the glycoprotein 60 (gp60) gene. Among calf samples, 14 samples were positive by MZN method. C. parvum was identified in all cases. Twelve parvum isolates (85.7%) belonged to family subtype IIa. Subtype IIaA15G2R1 was more prevalent (50%). Two C. parvum isolates corresponded to the IIdA16G1 subtype. Seven human samples were positive by MZN method. C. parvum and C. meleagridis were identified in four and three cases, respectively. Intraspecific characterization of C. parvum identified two subtypes, the IlaA15G2R1 and the IIdA16G1, also found in calves.

INTRODUCTION

The protozoan Cryptosporidium is a major public and animal health concern. Immuno compromised people, young children, and pre-weaning animals are especially vulnerable. Until now, there is no effective treatment or vaccine commercially available to prevent the disease. Currently, 26 Cryptosporidium species have been named, and there is good evidence for 6 species as important causes of human cryptosporidiosis: C. hominis, C. parvum, C. meleagridis and occasionally, C. cuniculus, C. felis, and C. canis. Despite occasional reports in livestock, C. hominis seems to be anthroponetically transmitted. The other species are zoonotic and mainly transmitted from animals to human. In animals, C. parvum and C. meleagridis are the most clinically and economically important gastrointestinal species in pre-weaning ruminants and birds, respectively. In dairy cattle, C. parvum is mostly found in pre-weaning calves, whereas three other species, including C. andersoni, C. bovis, and C. ryanae, are found in older age groups. In Tunisia, Cryptosporidium spp. was identified as a prevalent parasite in human and farm animals. C. hominis and C. parvum were the dominant species in urban residents, whereas C. parvum and C. meleagridis were the causative species in children from rural areas. C. bovis was found in lambs, and C. meleagridis was found in one broiler chicken. Until now, no data are available about identification of subtypes of Cryptosporidium species.

MATERIALS AND METHODS

Fecal specimens and sample sites. Cattle breeding in Tunisia is observed mainly in the northern areas of the country, where bovine farming is supported by favorable climatic conditions. Livestock statistics for 2006 show that 65% of the cattle are in the north and that more than 40% of the pure breed cattle population is found in the two northern districts of Beja and Bizerte. The study was performed in Bizerte District, which is located in northeast Tunisia (Figure 1). From April to October of 2007, 70 stool samples were collected immediately after defecation from calves less than 5 months in farms and private breeding units from the Jomine region (Figure 1). During the same period, 403 stool specimens were collected from children under 5 years of age; 258 stools (including 52 diarrheic specimens) were sampled from the pediatric rural communities (mean age = 33 months, SD = 17) living around farms animals of the Jomine region by a door to door survey, whereas 145 stools (including 51 diarrheic specimens) were collected in the healthcare unit of Menzel Bourguiba (mean age = 33 months, SD = 15), which is an important human settlement of Bizerte District (Figure 1).

Oocyst detection and species identification. Fresh stool specimens were examined for Cryptosporidium spp. oocysts.

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Microscopic examination was carried out on smears of fecal concentrates (simplified version of Ritchie’s formalin-ether sedimentation method)\textsuperscript{10} after staining with the modified Ziehl–Neelsen technique (MZN).\textsuperscript{11,12} DNA was extracted from all positive specimens using the QIAmp DNA Stool Mini-Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer’s recommendations. \textit{Cryptosporidium} species were identified using a two-step 18S rRNA nested polymerase chain reaction (PCR) followed by an RFLP analysis as described by Coupe and others\textsuperscript{5,13} with some modifications.

\textbf{Intraspecific characterization of} \textit{C. parvum} \textbf{isolates.} Subgenotyping of \textit{C. parvum} isolates was performed using nested PCR to amplify a fragment of the gp60 gene as described elsewhere.\textsuperscript{1,7} Briefly, two-step nested PCR was used. AL3531 (5′-ATAGTCTCCGCTGTATTC-3′) and AL3535 (5′-GGAAGGAACGATGTATCT-3′) primers were used for the first-round PCR and AL3532 (5′-TCCGCTGTATTCAGCC-3′) and AL3534 (5′-GCAGAACCAGCATC-3′) primers were used for the second-round PCR to amplify a 840-bp fragment. Extracted DNA (1 μL) was mixed with a solution containing 200 nmol each primer, 200 μM 2′-deoxynucleoside 5′-triphosphate, 1.5 mM MgCl\textsubscript{2}, and 2.5 U HotStar Taq polymerase (Qiagen GmbH, Hilden, Allemagne), with a final volume of 50 μL. Cycling conditions were an initial denaturation at 94°C for 10 minutes followed by 35 cycles of a three-step program (94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 1 minute). The amplified DNA fragments were purified using the Wizard Genomic DNA Purification Kit (Promega, Charbonnières, France) and sequenced in both directions with a Big Dye Terminator Cycle Sequencing Kit on an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA). For each isolate, the \textit{C. parvum} family group was assigned by sequence comparison with those isolates available in the GenBank database and published in peer-reviewed international scientific journals (www.ncbi.nlm.nih.gov/blast). Subtypes were named on the basis of the number of TCA (A), TCG (G), and ACATCA (R) as described by Sulaiman and others.\textsuperscript{14} and Gatei and others.\textsuperscript{7} Nucleotide sequences were aligned with reference genotypes from GenBank using ClustalW and analyzed using Mega5 software.

\textbf{RESULTS}

Among calf samples, 15 (21.4%) samples were positive by the MZN method. PCR confirmed positivity, and RFLP analysis yielded typical restriction patterns for \textit{C. parvum} in all cases. Intraspecific characterization of \textit{C. parvum} isolates identified only two subtype families: IIa and IId (Figure 2); 13 of 15 \textit{C. parvum} isolates (86.7%) belonged to family subtype IIa. The IIaA15G2R1 was the more prevalent (46.2%) subtype within this family (Table 1). The two latter \textit{C. parvum} isolates corresponded to the IIdA16G1 subtype (Table 1).

Among human samples, seven (1.7%) samples were positive by the MZN method. PCR confirmed positivity, and RFLP analysis yielded typical restriction patterns for \textit{C. parvum} in four cases and \textit{C. meleagridis} in three cases. Intraspecific characterization of \textit{C. parvum} isolates identified only two subtypes: IIaA15G2R1 in two cases and IIdA16G1 in two other cases (Table 1). \textit{Cryptosporidium} species and \textit{C. parvum}}
subtypes according to epidemiological data are reported in Table 2.

Phylogenic analysis of all *C. parvum* strains was shown in Figure 2.

**DISCUSSION**

Conventional procedures for oocyst concentration and detection in stool specimens, namely formalin-ether sedimentation and acid-fast staining, were used for *Cryptosporidium* screening. However, the high threshold necessary for oocyst detection by these coprodiagnosis methods, particularly in formed or semifomed stool specimens, could limit oocyst detection in asymptomatic individuals and underestimate the number of positive samples.15,16

Prevalence rate of *Cryptosporidium* spp. infection among children less than 5 years old was particularly low (1.7%). This rate may be partly because of the sample processing methodology but also could have been related to an absence of close contact between the human population sampled and animals. *C. meleagridis* was found almost as frequently as *C. parvum*. This finding suggests that zoonotic transmission from poultry is one of the most important causes of human crypto-sporidiosis in rural communities of Tunisia, where most families have their own poultry breeding close to their houses. However, even in this farming region, families do not necessarily have their own cattle, which suggest a low direct contact with these animals. Nevertheless, *C. parvum* was the only species identified in the positive calf stool specimens collected in the same region, which provides evidence of possible association of infected calves and human infection with *C. parvum*. The role of cattle in the zoonotic transmission of *C. parvum* in northern Tunisia was further supported by gp60 subgenotyping data.

![Figure 2. Phylogenic analysis of the gp60 nucleotide sequences of *C. parvum* strains from humans and calves (tree obtained by the method of UPGMA with *C. meleagridis* as an outgroup and bootstrap values based on 1,000 replicates). Our samples: E459, E473, E21, and E424 were from humans and VB2, V7, V9, V10, V4, V17, V18, VB7, VB8, V11, VB1, V14, VB22, and VB24 were from calves. Other nucleotide sequences were from GenBank.](image)

<table>
<thead>
<tr>
<th>Study area and code</th>
<th>Age group (years)</th>
<th>Sex</th>
<th>Stool consistency</th>
<th>Cryptosporidium species</th>
<th><em>C. parvum</em> subtype family</th>
<th><em>C. parvum</em> subtypes</th>
</tr>
</thead>
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<tr>
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<td>A16G1</td>
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<td>Menzel Bourguiba region</td>
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</table>

D = diarrheic; F = formed.

<table>
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<tr>
<th>Subtype family and subtype</th>
<th>Number of isolates in humans</th>
<th>Number of isolates in calves</th>
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<tr>
<td>A16G1</td>
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</tr>
</tbody>
</table>

**Table 1** *Cryptosporidium* species and *C. parvum* subtypes identified in human population according to epidemiological data

**Table 2** *C. parvum* subtypes identified in calves from Bizerte District

D = diarrheic; F = formed.
As reported by other studies, family subtype IIa was the dominant family in calves. Also, the IIA15G2R1 subtype seemed to be the most common subtype on the dairy farms. Interestingly, two of four (50%) C. parvum isolates from children belonged to family subtype IIa and were similar to the IIA15G2R1 subtype (the most prevalent subtype identified in calves during the same period), which suggests that family subtype IIa and particularly, IIA15G2R1 subtype can spread easily within cattle populations and be transmitted to humans as well. In fact, this variant is frequently observed in C. parvum populations worldwide and has been widely reported in zoonotic infections. As a risk factor for human cryptosporidiosis, contact with cattle was implicated in the neighboring countries, such as Egypt and Spain, as well as the United States, United Kingdom, Ireland, and Australia. However, in our study, taking into account the age of children and their kind of life (human settlement), contact with livestock is possible; however, drinking raw (unpasteurized) milk may also represent a probable risk factor for Cryptosporidium transmission in this area. More investigations should be performed with large and more representative samples.

As reported in European countries, the IId subtype family was occasionally found in calves in addition to IIa subtypes. In this study, the IIdA16G1 was identified in two calves as well as two children living in the rural environment around farm animals. Interestingly, IId subtypes of C. parvum have never been found in humans in the United States and Canada, where they are absent in calves. Thus, the less common bovine C. parvum subtype family IId may potentially also be responsible for some zoonotic infections in northern Tunisia. However, the relatively high proportion of cases (50%) in humans highlights the need for additional epidemiological investigations. In fact, in regions where both subtypes IIa and IId are found (i.e., Spain), family subtype IIa infects preferentially calves, whereas family subtype IId has a tropism for lambs and kids. Although an earlier study conducted in Tunisia suggested that C. parvum was absent in sheep, studies involving more animal samples from lambs are needed for a better understanding of the sources of Cryptosporidium human infections in this northern area.

Received September 10, 2013. Accepted for publication September 30, 2013.

Published online December 16, 2013.

Acknowledgments: We are grateful to Dr. R. Hamza and all the staff of the Regional Directory of Public Health of the Bôuez governorate for their contribution to the achievement of this work. We also thank Dr. R. Ben Omrane, Dr. M. Sayari, and H. Abassi for their help in calf stool collections and Dr. S. Benabderrazak for her help in phylogenetic analysis.

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